

Growth of Paramecia in Pure Cultures of Pathogenic Bacteria and in the Presence of Soluble Products of Such Bacteria

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GROWTH OF PARAMECIA IN PURE CULTURES OF PATHOGENIC BACTERIA AND IN THE PRESENCE OF SOLUBLE PRODUCTS OF SUCH BACTERIA ¹

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SIX CHARTS

AUTHOR'S ABSTRACT

Virulent hay-infusion cultures of *Bacillus pyocyaneus* are toxic to pure-line races of three species of paramecia, but these races may acquire a tolerance for this toxic agent. Races with acquired tolerance have been grown for long periods of time in toxic, pure cultures of *B. pyocyaneus* by means of the daily-isolation culture method, and here the average division rate is as high as, or higher than, in the chance-mixed bacterial cultures in which these protozoa are usually maintained in the laboratory. The tolerance is lost, however, when the paramecia are removed from the toxic cultures and grown for a number of generations in cultures of non-toxic bacteria.

The toxic agent that is lethal to paramecia is probably the soluble toxin of *B. pyocyaneus*. The investigation shows that the agent is soluble and either thermolabile or volatile. It also shows that all deleterious substances, other than the soluble toxin, known to be produced in cultures of this bacillus, are non-lethal to paramecia.

Hay-infusion cultures of *Bacillus enteritidis* were lethal to paramecia. All attempts to develop tolerance in paramecia for the toxic agent in these cultures failed.

Under the experimental conditions that prevailed, diphtheria toxin was found to have no appreciable effect upon the division rate or death rate in three species of paramecia.

INTRODUCTION

In this initial investigation of the effect of pathogenic bacteria and bacterial products upon protozoa the first problem at hand was that of selecting materials with which to work. Paramecium was chosen because of the ease with which it may be grown under laboratory conditions. Moreover, it can be grown in pure cultures of bacteria, as has been shown by Hargitt and Fray ('17) and Phillips ('22). *Bacillus pyocyaneus* and *Bacillus enteritidis* were selected because they are pathogenic and are able to grow luxuriantly in the hay-infusion media that are commonly used for paramecia. It was also the desire to select some standardized toxin whose effect upon paramecia could be studied. A number of toxins

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are available for such a study, including those of bacterial origin as well as those produced by higher plants and animals. In the present investigation, however, only one standard toxin, that of the diphtheria bacillus, has been used.

The investigation has been exploratory in its nature. In the main it was the intention to study the effect of these pathogenic bacteria and bacterial products upon paramecia and to determine the extent to which races of paramecia are able to adapt themselves to the deleterious effects of these agents. It was also hoped that the study might eventually yield data of significance in connection with certain fundamental questions in pathology and immunology. It was felt that the study might incidentally offer an opportunity to extend the technique of Hargitt and Fray ('17) and of Phillips ('22) in methods of growing paramecia in pure cultures of bacteria. There was also the opportunity to continue the search, initiated by these investigators, for a species of bacteria in pure cultures of which paramecia may be grown successfully.

The present investigation has been in progress since the spring of 1924. Most of the work has been carried on at the Marine Biological Laboratory, Woods Hole, Massachusetts, and at the Zoölogical Laboratory of the University of Missouri. The study has been made possible largely through the encouragement and help of Prof. W. C. Curtis, of the University of Missouri, and through the facilities of his laboratory. To Prof. L. L. Woodruff, of Yale University, thanks are also due for his interest during the summer of 1925. For assistance and for materials and apparatus thanks are due Dr. Mazyck P. Ravenel, of the Department of Medical Bacteriology, and to Prof. W. J. Robbins, of the Department of Botany, both of the University of Missouri.

MATERIALS AND METHODS

Materials

Bacillus pyocyaneus Gessard (*Pseudomonas aeruginosa* Schroter Migula) and *Bacillus enteritidis* Gaertner (Salmon-

ella enteritidis Gaertner) were selected as the pathogenic bacteria to be used in this investigation, but only the former proved to be favorable. To make the work more comprehensive, experiments were conducted with three species of paramecia, *Paramecium aurelia* and *P. calkinsi*, from Prof. L. L. Woodruff's pedigreed stocks maintained at Yale University, and *P. caudatum* from wild strains. In each experiment the controls and experimental animals were obtained from a single pure line established for the purpose.

Diphtheria toxin was selected as a soluble toxin to be used in the study, because of its availability and standardization. Time has not permitted the extension of the study to other standard toxins.

Further explanations of the materials used appear in connection with the accounts of particular experiments.

Culture media

The standard hay infusions used in this study were made by adding 10 grams of chopped timothy hay to a liter of water and boiling for five minutes. This is referred to throughout this report as '0.2 per cent standard hay infusion.' Ordinary distilled water was used in some experiments. In others tap-water was used. The kind of water used is specified for each experiment. Enough hay infusion was made each time to last throughout the experiment and this was placed in 100-cc. Erlenmeyer flasks, that were then plugged with cotton and sterilized in an Arnold sterilizer by the intermittent method. The pH of each infusion was obtained after sterilization and recorded for each experiment.

Method of washing the paramecia bacteria-free

To grow paramecia in pure cultures of bacteria it is, obviously, necessary to free the animals from miscellaneous bacteria attached to their surface. In the present work animals were sterilized by a method which differed very slightly from those devised by Hargitt and Fray and by Phillips. Each

animal to be freed from bacteria was transferred by means of sterile pipettes through eight sterile wash waters. The first two wash waters were contained in culture slides in a sterile Petri dish. These slides had deep cylindrical cavities each containing approximately 0.5 cc. of liquid, which gave the animal a greater volume of sterile medium and thus facilitated sterilization. The last six waters through which each animal was passed were contained in watch crystals, as in the work of Phillips. Animals were transferred in each case under the low power of a binocular, and in each transfer the lid of the Petri dish was raised barely enough to permit the entrance of the capillary pipette. The large end of each pipette was plugged with cotton to prevent contamination from the rubber bulbs which were not sterilized. As a fresh sterile pipette was used for each transfer from one sterile water to another, it was necessary to have them on hand in large numbers. Ground-glass-stoppered specimen jars were used as containers, each containing as many as twenty-five pipettes. These were sterilized in hot air. These and other standard forms of technique used by bacteriologists were applied throughout the work.

Efficiency of this method

Tables 1 and 2 show the efficiency of the foregoing method of sterilizing paramecia. Nineteen individuals of *P. caudatum* and fourteen of *P. aurelia* were washed, and in each case a record was made to show, *a*) whether the water in which the animal was washed was sterile (column 7); *b*) whether the last two wash waters remained sterile after the washing (columns 2 and 3); *c*) whether each washed animal when introduced into 0.5 cc. of sterile hay water continued to live more than twenty-four hours (columns 4 and 5), and, *d*) whether the sterile water into which the washed animals were introduced remained sterile (column 6). The sterility of this water was determined in each case by placing a portion of it on the surface of an agar slant and incubating the tube at 22°C. for two weeks. In this manner a total of thirty-three

animals were washed in the order shown in tables 1 and 2, and no cases were omitted.

From the data in tables 1 and 2 it may be concluded that a paramecium can be completely sterilized by the method above described. In table 1, animals 5, 6, 9, 10, 13, 14, 15, and

TABLE 1

Efficiency of washing nineteen individuals of Paramecium caudatum. The animals are listed in the order in which they were washed, and no cases are omitted

(1) NO.	(2) 7TH WASH	(3) 8TH WASH	(4) CONDITION OF ANIMAL 24 HOURS AFTER WASHING	(5) CONDITION OF ANIMAL 48 HOURS AFTER WASHING	(6) CONDITION OF WATER 48 HOURS AFTER WASHING	(7) CONTROL WASH WATER
1	Sterile	Sterile	One alive	One alive	Contaminated	Sterile
2	Sterile	Sterile	One alive	One alive	Contaminated	Sterile
3	Sterile	Sterile	One alive	One alive	Contaminated	Sterile
4	Sterile	Sterile	(Animal died in 7th wash)			
5	Sterile	Sterile	Dead	Dead	Sterile	Sterile
6	Sterile	Sterile	Dead	Dead	Sterile	Sterile
7	Sterile	Sterile	Dead	Dead	Contaminated	Sterile
8	Sterile	Sterile	Dead	Dead	Contaminated	Sterile
9	Sterile	Contaminated	Dead	Dead	Sterile	Sterile
10	Sterile	Sterile	Dead	Dead	Sterile	Sterile
11	Contaminated	Contaminated	Dead	Dead	Contaminated	Sterile
12	Sterile	Sterile	Dead	Dead	Contaminated	Sterile
13	Sterile	Sterile	Dead	Dead	Sterile	Sterile
14	Sterile	Sterile	Dead	Dead	Sterile	Sterile
15	Sterile	Sterile	Dead	Dead	Sterile	Sterile
16	Sterile	Contaminated	Dead	Dead	Sterile	Sterile
17	Contaminated	Sterile	One alive	One alive	Contaminated	Sterile
18	Sterile	Contaminated	One alive	One alive	Contaminated	Sterile
19	Sterile	Sterile	One alive	Two alive	Contaminated	Sterile

16 were successfully sterilized. In table 2, animals 1, 3, 6, 7, 10, 11, 12, and 14 were successfully sterilized. Out of a total of thirty-three animals washed, sixteen were rendered completely bacteria-free.

The data make it necessary to conclude, however, that the method cannot be relied upon to successfully sterilize every animal washed. Animals 1, 2, 3, 4, 7, 8, 11, 12, 17, 18, and 19

in table 1, and 2, 4, 5, 8, 9, and 13 in table 2 were not sterile, for in each case the hay water in which the animal died showed bacterial contamination.

Incidentally, the results shown in the above study throw some light upon the length of time paramecia will live in sterile media. Combining the results obtained with *P. cau-*

TABLE 2

Efficiency of washing fourteen individuals of Paramecium aurelia. The animals are listed in the order in which they were washed, and no cases are omitted.

Animals 12 and 14 lived three days after washing

(1) NO.	(2) 7TH WASH	(3) 8TH WASH	(4) CONDITION OF ANIMAL 24 HOURS AFTER WASHING	(5) CONDITION OF ANIMAL 48 HOURS AFTER WASHING	(6) CONDITION OF HAY WATER 48 HOURS AFTER WASHING	(7) CONTROL WASH WATER
1	Sterile	Sterile	Dead	Dead	Sterile	Sterile
2	Sterile	Sterile	One alive	One alive	Contaminated	Sterile
3	Sterile	Contaminated	Dead	Dead	Sterile	Sterile
4	Sterile	Sterile	Dead	Dead	Contaminated	Sterile
5	Sterile	Sterile	Dead	Dead	Contaminated	Sterile
6	Sterile	Sterile	Dead	Dead	Sterile	Sterile
7	Sterile	Sterile	Dead	Dead	Sterile	Sterile
8	Contaminated	Contaminated	Dead	Dead	Contaminated	Sterile
9	Sterile	Sterile	Dead	Dead	Contaminated	Sterile
10	Sterile	Sterile	Dead	Dead	Sterile	Sterile
11	Sterile	Sterile	Dead	Dead	Sterile	Sterile
12	Sterile	Sterile	One alive	One alive	Sterile	Sterile
13	Sterile	Sterile	One alive	One alive	Contaminated	Sterile
14	Sterile	Sterile	One alive	One alive	Sterile	Sterile

datum and those with *P. aurelia*, it may be said that out of sixteen cases of successful sterilizations (nos. 5, 6, 9, 10, 13, 14, 15, and 16 of table 1 and nos. 1, 3, 6, 7, 10, 11, 12, and 14 of table 2) only two animals (nos. 12 and 14 of table 2) were able to survive complete inanition more than twenty-four hours and that the maximum duration of life for these two was three days.

GROWTH OF PARAMECIA IN PURE CULTURES OF BACILLUS
PYOCYANEUS*Material*

It was desirable to use in this study an organism that is known to form a soluble toxin, in addition to being pathogenic to many animals. It was necessary that this organism should be one that grows well in hay water and preferably one that is not dangerously pathogenic to man. *Bacillus pyocyaneus* meets these requirements, although there is some difference of opinion concerning the seriousness of infections by this bacillus in man. The use of this species was suggested by Dr. Moyer S. Fleischer, of the Saint Louis University Medical School, and the bacteria used throughout the study came from his stock culture of this organism. Examinations made from time to time have shown that the organism is Gram negative and motile. It produces in agar the typical pigment of *B. pyocyaneus* and conforms in its growth in milk and in the various sugar media to the descriptions given for this bacillus in Bergy's Manual.² The pathogenicity of the organism was determined, after the culture had been maintained under laboratory conditions on agar for sixteen months, by injecting 0.5 cc. of a heavy suspension of the living organisms in physiological salt solution into each of three guinea-pigs. It was found that either subcutaneous or intraperitoneal injections in every case killed the animal in less than twelve hours. In one case the organism was recovered in pure culture from the subcutaneous exudate, from the peritoneal cavity, and from the heart-blood. Death of guinea-pigs resulting from such injections as described above was interpreted by Kolle and Wasserman ('03) as being due to the action of endotoxin.

The hay infusions containing the pure cultures of *Bacillus pyocyaneus* were prepared by inoculating sterile 0.2 per cent hay water contained in 100-cc. Erlenmeyer flasks and incubated at 35°C. for two days. These flasks were only partially

² Bergy, David H. Manual of Determinative Bacteriology, 1923, pp. 123, 124.

filled, in order to provide a greater amount of surface for aeration. The hay water was prepared by the method previously described. In each case a large quantity of the medium was prepared in one large container to insure uniformity of composition, and then placed in a number of cotton-plugged flasks to be sterilized in steam by the intermittent method. This arrangement provided in each case enough hay infusion of uniform composition for at least five weeks. The pH of all media made by this method ranged from 6.8 to 8.3. Very little difference was found between the pH of sterile hay water and two-day cultures of *B. pyocyaneus* made

TABLE 3

Hydrogen-ion concentration of sterile hay water and of cultures made from it of B. pyocyaneus and of chance-mixed bacteria from the air

DATES	AUGUST 15, 1925	AUGUST 10, 1926
Methods	Colorimetric	Hydrogen electrode potentiometer
Sterile hay water	6.8	8.14
Two-day culture of <i>B. pyocyaneus</i>	7.0	8.23
Ten-day culture of <i>B. pyocyaneus</i>	7.0	
Twenty-four-hour chance-mixed bacterial culture in same hay infusion	6.8	8.0

with the same hay infusions. The data in table 3 give the results of pH determinations made at two different times. The infusions used in all experimental work carried on during the months of June, July, and August, 1925, were made from Woods Hole pond water. From September, 1925, to June, 1926, St. Louis tap-water was used. During June, July, and August, 1926, infusions were made from distilled water and from St. Louis tap-water.

Methods

In establishing the growth of a pure line of paramecia in pure cultures of *B. pyocyaneus* a single animal was first isolated and permitted to undergo one division. Of the daughter

cells, one was used as the progenitor of all lines grown throughout the course of the experiment in the cultures of *B. pyocyaneus*; the other was used as the progenitor of all lines grown as controls in the chance-mixed bacterial cultures. The progenitor of lines to be grown in the pure cultures of *B. pyocyaneus* was washed by the method already described and then introduced by means of a sterile pipette into 0.5 cc. of the pure two-day culture of *B. pyocyaneus* contained in a sterile culture slide in a Petri dish. The immediate offspring of this animal were used as the progenitors of as many lines as was necessary to continue in the pure cultures. In each line that was thus continued transfers were made daily to fresh two-day cultures of bacteria. Animals were transferred in every case with sterile pipettes. Each transfer was made under a binocular and in each operation the lid of the Petri dish was raised barely enough to admit the pipette. After these lines had grown for a few days in the pure cultures, it was necessary to determine whether the cultures were still pure or whether foreign bacteria had been introduced with the washed paramecium or by any means. To do this a drop of each culture from each line, taken in each case after paramecia had grown in it for twenty-four hours, was plated out in agar by the usual bacteriological method. Where the cultures showed contamination, that line was discontinued and replaced by another started from one of the lines showing no contamination. Throughout the course of each experiment a record was kept for each line of the number of divisions per day and the number of deaths.

Experiments and observations

Paramecia grow at a high division rate and for apparently an indefinite period of time in pure cultures of *Bacillus pyocyaneus*. This occurs even in toxic cultures of *B. pyocyaneus*, provided the races of paramecia are first made tolerant to the toxic agent in the cultures. This fact has some significance in the light of certain discussions during recent years as to whether paramecia can maintain themselves in pure

cultures of any species of bacterium. Hargitt and Fray ('17), working with eleven of the common species of bacteria which inhabit hay infusions, found that paramecia did not maintain as high a division rate in pure cultures as when the animals grew in bacterial mixtures. Phillips ('22) found the same to be true for the twelve additional species that she studied.

The growth of four lines of *P. aurelia* for one hundred days in hay-infusion cultures of *B. pyocyaneus* and in chance-mixed bacterial cultures, similarly made with hay infusions, is summarized in figure 1 and in table 4. Since the cultures of *B. pyocyaneus* used were found to be toxic to *P. aurelia*, it was necessary to habituate the races to this toxic action before beginning the experiment. The method of such habituation is described and discussed in a later section (compare p. 99). The mean division rate of the four lines in the cultures of *B. pyocyaneus* for the one hundred days was 1.52, while that for the lines in chance-mixed cultures was 1.22, thus showing a higher division rate in the cultures of *B. pyocyaneus*.

So far as possible, the four lines of *P. aurelia* grown in cultures of *B. pyocyaneus* and the four control lines in the mixed-bacterial cultures were kept under uniform conditions. All lines were pedigreed stock having descended from one animal at the beginning of the hundred-day period. Conjugation was eliminated by the use of the daily isolation method. The temperature was controlled to between 22° and 25°C. The light was variable, but affected all lines similarly. The hay infusions used had a pH (determined colorometrically) of from 8.2 to 8.5 when sterile. Cultures of *B. pyocyaneus* made with this hay water did not develop any appreciable change in acidity or alkalinity. The pH of the chance-mixed bacterial cultures made from the hay water ranged from 7.1 to 8.0.

The growth of a number of lines of *P. caudatum* for fifty-five days in *B. pyocyaneus* and in chance-mixed bacterial cultures is summarized in figure 2 and in table 4. Here also the division rate is higher in the cultures of *B. pyocyaneus*, the

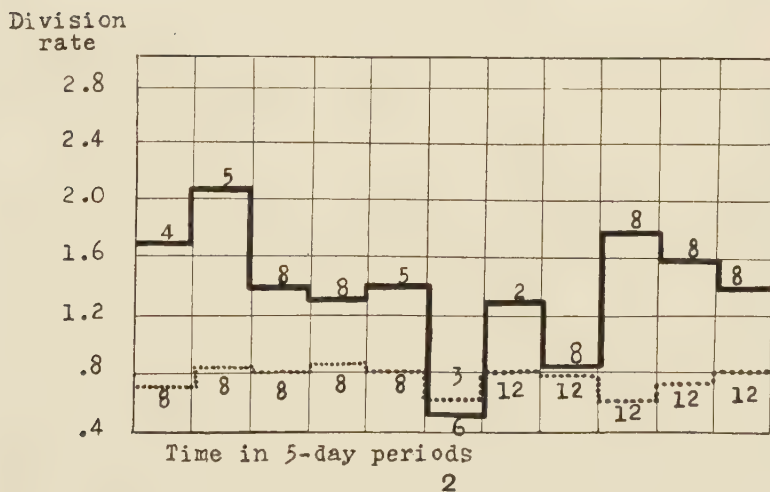
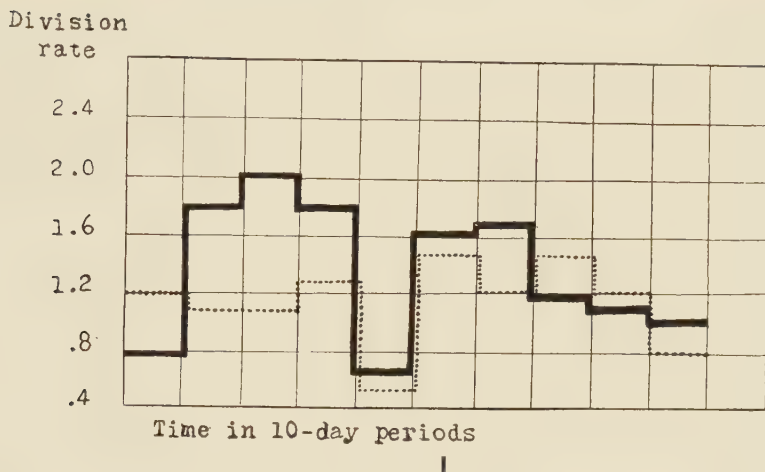


Fig. 1 Growth of four lines of *P. aurelia* for one hundred days in two-day cultures of *B. pyocyaneus* (heavy line) and in chance-mixed bacterial cultures (dotted line). February 26 to June 5, 1926. Each curve represents the average division rate of four lines again averaged for ten-day periods. Temperature, 22° to 25°C.

Fig. 2 Growth of a varying number of lines of *P. caudatum* for fifty-five days in two-day cultures of *B. pyocyaneus* (heavy line) and in chance-mixed bacterial cultures (dotted line). July 3 to August 26, 1925. Each curve represents the average division rate of a varying number of lines again averaged for five-day periods. The figures on the curves represent the number of lines of paramecia averaged into each five-day period. No control of temperature.

mean division rate being 1.4 as compared with 0.75 in the chance-mixed cultures. The same methods were used as for *P. aurelia*, except that there was no control of the temperature. The number of lines as shown in figure 2 varied from week to week. During most of the time eight lines were maintained, but during a few weeks the number was increased to twelve, and at the beginning of the experiment and later during periods of depression reduced to a smaller number. Each line was descended from one individual isolated at the

TABLE 4

Comparison of the mean division rates of three species of paramecia in hay-infusion cultures of B. pyocyaneus and of chance-mixed bacteria from the air

	MEDIUM	NUMBER OF DAYS	MEAN DIVISION RATE	PROBABLE ERROR	DIFFERENCE IN MEAN DIVISION RATES	PROBABLE ERROR OF DIFFERENCE	SIGNIFICANCE FACTOR
P. Aurelia	A. Mixed cultures	100	1.22	.50			
	B. Cultures of <i>B. pyocyaneus</i>	100	1.52	.53	.30	.73	.41
P. Caudatum	A. Mixed cultures	55	.75	.06			
	B. Cultures of <i>B. pyocyaneus</i>	55	1.4	.25	.65	.25	2.5
P. Calkinsi	A. Mixed cultures	85	1.48	.36			
	B. Cultures of <i>B. pyocyaneus</i>	85	2.54	.42	1.06	.55	1.9

beginning of the fifty-five-day period. These cultures of *B. pyocyaneus* were also toxic, and it was necessary, as with *P. aurelia*, to habituate all the lines of *P. caudatum* grown in them. The method of habituation is discussed later (compare p. 100).

The 0.2 per cent timothy-hay infusion used during the fifty-five-day period was made from Woods Hole pond water, and the animals used came from one individual isolated from a Woods Hole pond. The pH of the sterile timothy-hay infusion and of the *B. pyocyaneus* and mixed cultures made from it is shown in table 3 under the date of August 15, 1925.

A race of *P. calkinsi* grown in the cultures for a period of eighty-five days gave results similar to those for *P. aurelia* and *P. caudatum*. The results for this race are shown in figure 3 and in table 4. The mean division rate in the cultures of *B. pyocyaneus* was 2.54, while that in the chance-mixed cultures was 1.48.

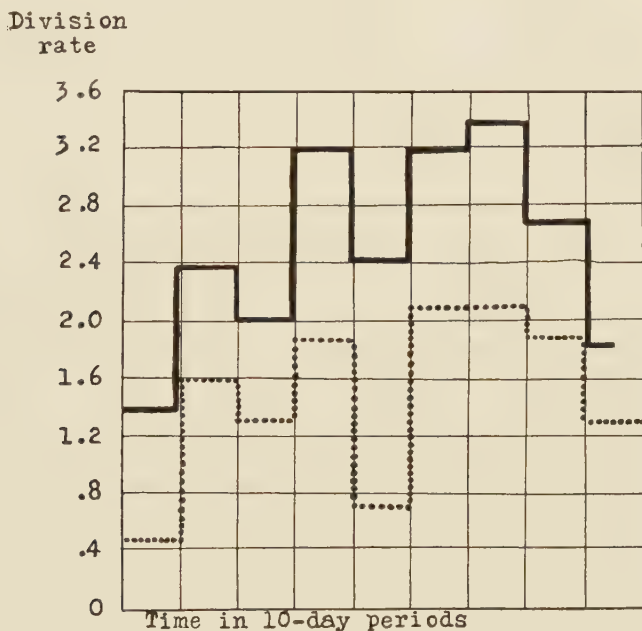


Fig. 3 Growth of four lines of *P. calkinsi* for eighty-five days in two-day cultures of *B. pyocyaneus* (heavy line) and in chance-mixed bacterial cultures (dotted line). May 29 to August 22, 1926. Each curve represents the average division rate of four lines again averaged for ten-day periods. No control of temperature.

The 0.2 per cent hay infusions used for *P. calkinsi* during the eighty-five-day period were made from distilled water and contained 0.04 per cent NaCl. Woodruff ('21) and others have found the presence of salt to be necessary for the growth of this species. The temperature during the period was controlled to between 24° and 28°C. The pH of the infusion used is recorded in table 3 under the date of August 10, 1926.

The cultures of *B. pyocyaneus* made from the distilled-water hay infusions were not toxic for *P. calkinsi*. The possible significance of this is discussed in a later part of this report.

With the three races of paramecia used it is evident that the division rate in the cultures of *B. pyocyaneus* is higher than in the mixed cultures. When, however, the probable error for each mean division rate is taken into consideration, the significance factors, as shown in table 4, do not indicate that the higher mean division rates in the cultures of *B. pyocyaneus* are of as much significance as one might at first think. The method of determining this significance factor is that used by Phillips ('22). It is based upon the mathematical assumption that the numerical value representing the difference in the means under two experimental conditions is of significance if it contains the probable error of the difference more than three times. The significance factors shown in table 4 indicate that with no one of the three races of paramecia was the higher division rate in the cultures of *B. pyocyaneus* of such significance. The fact remains, however, that the average division rate in the cultures of *B. pyocyaneus* was at least as high as in the chance-mixed bacterial cultures, and this fact is significant in connection with the question whether pure cultures of bacteria can support continued growth of paramecia as well as chance-mixed cultures.

It may be possible, however, that the periods of high division rate of paramecia in the cultures of *B. pyocyaneus* is due to the stimulation by a toxic substance rather than to the superior nutritive qualities of these cultures for paramecia. Evidence is presented in another part of this report to show that an agent, which is toxic to paramecia, is present in hay-water cultures of *B. pyocyaneus*. It is a well-known fact that many poisonous substances may either retard or accelerate the general activity of protoplasm, depending upon the amount of the agent present (Pfeffer, '03, p. 264).

DEVELOPMENT BY PARAMECIA OF TOLERANCE TO THE TOXIC EFFECTS OF CULTURES OF *BACILLUS PYOCYANEUS*

Cultures of *B. pyocyaneus* made from 0.2 per cent hay water, prepared by diluting standard 1 per cent hay infusion with tap-water, were found to be toxic for *P. aurelia*, *P. caudatum*, and *P. calkinsi*; but under certain conditions races of these animals acquired a tolerance to this toxic action. This may be illustrated by the results obtained with *P. aurelia* (fig. 4). In this experiment a race, consisting of four lines descending from one animal isolated from Woodruff's stock culture of *P. aurelia*, was made tolerant to the toxic effects of two-day cultures of *B. pyocyaneus* by growing lines in the toxic cultures for a period of four days and then returning them for a period of recovery to chance-mixed bacterial cultures. After this four-day recovery period, the lines were again returned to the toxic cultures in which they were then able to grow during the remainder of the one hundred days of the experiment. Where no recovery period was provided, the lines introduced into the toxic cultures invariably died out. The heavy unbroken marking in figure 4 shows the average daily division rate during the hundred-day period of the lines in which tolerance had been acquired. Four control lines, also descended from the one individual isolated at the beginning of the experiment, were grown throughout the experiment in chance-mixed bacterial cultures. The average division rate of these controls is shown by the dotted marking. The toxic effect of *B. pyocyaneus* cultures was tested from time to time by introducing four lines of the animals taken from the controls into the toxic cultures. The fate of all of these test lines in which tolerance had not been acquired is shown in the figure. Each marking of dashes represents the average division rate of four of these test lines. In all, forty test lines were used, and no one of them was able to resist the toxic action, although the four lines in which tolerance had been acquired maintained a high division rate in the cultures of *B. pyocyaneus* throughout the entire period. Care was taken that the cultures of *B. pyo-*

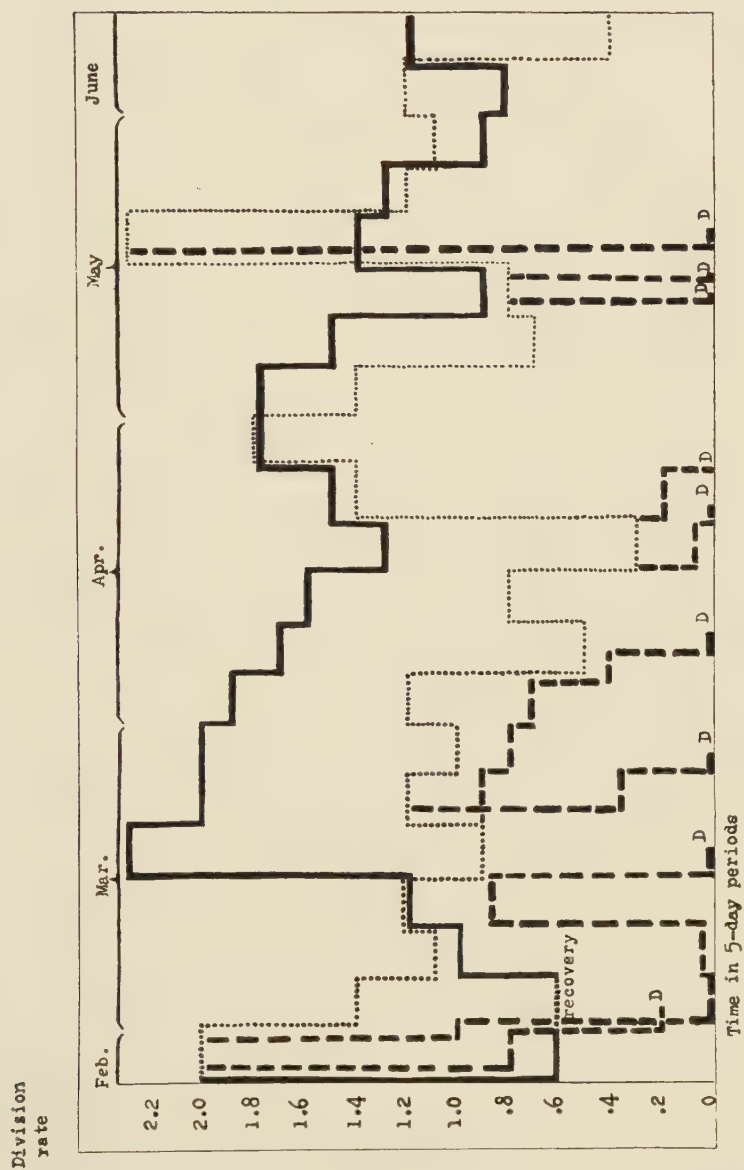
cyaneus used for each test line were removed from the same flask as used that day for the four tolerant lines. In other words, there was no controllable difference at any time between the environment of the lines in which tolerance had been acquired and that of the non-tolerant test lines. The medium used during the hundred-day period was 0.2 per cent timothy-hay infusion made from St. Louis tap-water. More recent experiments, discussed in a later part of this report, show that distilled water cannot be used in making a hay-water culture of *B. pyocyaneus* that will be toxic to *P. aurelia*. The temperature of the experiment was controlled to within two degrees of 20°C.

It is clear that cultures of *B. pyocyaneus* were similarly toxic to *P. caudatum* and that tolerance was also acquired, but no attempt was made to collect as much evidence to show this as was collected for *P. aurelia*. In fact, *P. caudatum* was the species first used in the experiments, and with it the first indications of toxic action were observed. This was in May, 1925, when repeated attempts were made, without success, to grow lines of *P. caudatum* in two-day cultures of *B. pyocyaneus*. In June, 1925, an attempt was made for the first time to render a line of *P. caudatum* tolerant to the toxic action of cultures of *B. pyocyaneus* by providing periods

Fig. 4 Development of tolerance by *P. aurelia* to the toxic effects of two-day cultures of *B. pyocyaneus*. The heavy marking represents the average division rate in two-day cultures of *B. pyocyaneus* of four lines of *P. aurelia* which had acquired tolerance for the toxic effects of these cultures. Tolerance was acquired during the second five-day period, at which time the lines were grown for recovery in chance-mixed bacterial cultures. These lines were averaged for five-day periods.

The dotted marking represents the division rate of four control lines which were grown in chance-mixed bacterial cultures. These were sister lines to those grown in the cultures of *B. pyocyaneus*. The marking of dashes represents the fate of non-tolerant test lines of *P. aurelia* which were taken from the control lines and introduced into two-day cultures of *B. pyocyaneus*. Each line of dashes represents the average division rate of four of these non-tolerant lines on those particular days.

All animals used were descendants of one individual isolated at the beginning of the experiment. The temperature was controlled constantly to within two degrees of 20°C.



of recovery in chance-mixed bacterial cultures. Success came so quickly that it was not recognized as such. The lines treated began to divide rapidly in the toxic cultures and continued to do so for a period of fifty-five days, at the end of which time it was necessary to discontinue the study. The ease with which this tolerance was acquired weakened belief in the toxic action of the pyocyaneus cultures upon paramecium, and in a preliminary report of the investigation made (Philpott, '25) I stated that only the ten-day, and not the two-day cultures, were toxic. Later studies with *P. aurelia* and *P. calkinsi* as well as further studies with *P. caudatum* confirm the first conclusion, that even two-day cultures of *B. pyocyaneus* are toxic and that races of paramecia may acquire tolerance to the toxic agent.

Some data obtained with *P. caudatum*, which indicate the toxic action of the cultures of *B. pyocyaneus* and the ability of this species to acquire tolerance are represented in figure 5. The heavy marking (5, A) represents the division rate of eight lines of *P. caudatum* in ten-day cultures of *B. pyocyaneus*. Previous to this time, these lines had grown for thirty days in chance-mixed bacterial cultures. These eight lines, as shown in the figure, grew well in the cultures of *B. pyocyaneus* for a period of ten days and then died out, with the exception of one line. This one line was saved by growing it for a five-day period in mixed bacterial cultures. It is the belief that this five-day recovery period brought about the development of tolerance in the animals. At the end of this recovery period an animal was returned to the ten-day cultures of *B. pyocyaneus*, and from its immediate progeny eight lines were produced which, as shown in the diagram, continued at a high division rate in the toxic cultures. The lines averaged in 5, B were sister lines to those shown in 5, A. These were tolerant lines and had previously grown for thirty days in cultures of *B. pyocyaneus*. These eleven tolerant lines, increased to twelve during the second five-day period and later to sixteen, did not show any depression in the division rate during the third five-day period when fatalities occurred in the non-tolerant lines shown in A.

All controllable factors in the lines that had been made tolerant and those not so treated were the same throughout the period of the experiment. The work was performed at Woods Hole during the summer of 1925. Hay water of 0.2 per cent, made from Woods Hole pond water, was used. More recent experiments, discussed in a later part of this



Fig. 5 (A) The heavy marking represents the division rate of eight lines of *P. caudatum* in ten-day cultures of *B. pyocyaneus*. These lines had previously grown in mixed-bacterial cultures. The dotted line represents the division rate of the controls in chance-mixed bacterial cultures. (B) The heavy marking represents the division rate of eleven to sixteen lines of *P. caudatum* in ten-day cultures of *B. pyocyaneus*. These were sister lines to those represented in (A), but had previously grown for thirty days in cultures of *B. pyocyaneus*. All division rates are averaged for five-day periods.

report, show that distilled water cannot be used in making a hay-infusion culture of *B. pyocyaneus* which is toxic to *P. caudatum*. All media used in this experiment were made up at the beginning of the fifty-five-day period in one large container and distributed in 100 cc. cotton-plugged flasks. Hence there could have been no variation in media from day to day. Each day the culture of *B. pyocyaneus* used for the

two sets of lines came from the same flask. There was no temperature control, but temperature variations were the same for all lines.

Under certain conditions, results have been obtained with *P. calkinsi* similar to those for *P. aurelia* and *P. caudatum*. Two-day cultures of *B. pyocyaneus* made from St. Louis tap-water were toxic to these animals, but they acquired tolerance

TABLE 5

Development of tolerance in four lines of P. calkinsi to growth in two-day cultures of B. pyocyaneus. The figures for each day indicate the number of divisions in the different lines for that day. The four lines in (A) were grown in two-day cultures of B. pyocyaneus except on certain days, as indicated by parentheses, when the animals were grown in chance-mixed bacterial cultures. All lines in (B) were grown daily in two-day cultures of B. pyocyaneus and there were no recovery periods in mixed cultures. When a death occurred in any line, a reserve animal in that line, from the preceding day when available, was used to take its place

MAY				JUNE										
	29	30	31	1	2	3	4	5	6	7	8	9	10	11
(A)	1	(1)	(2)	1	1	2	0	0	2	2	1	2	3	3
	1	(0)	(2)	(2)	2	1	(1)	1	2	1	3	2	3	3
	1	(1)	(2)	(1)	2	1	(0)	1	2	1	3	3	2	2
	1	(0)	(3)	(1)	2	(1)	1	1	3	1	3	2	2	2
(B)	1	1	3	2	2	D								
	1	D	2	2	2	2	0	D						
	1	D	2	2	2	2	0	D						
	1	2	1	2	3	2	0	D						

to the toxic agent as shown in table 5. The eight lines shown here were descendants of one animal. Four lines of animals (table 5, A) were made tolerant by growing in two-day cultures of *B. pyocyaneus* and providing periods of recovery in mixed bacterial cultures. The recovery periods are indicated by figures in parentheses. The four sister lines (table 5, B) were grown under the same conditions as for the four tolerant lines (table 5, A), except that no recovery periods were provided. These lines (5, B) were not able

to develop tolerance to the toxic agent in the culture, and all were dead at the end of the eighth day. As in previous experiments, the cultures of *B. pyocyaneus* used for all lines during any one day came from one flask. The hay infusion used was made from St. Louis tap-water to which 0.2 per cent NaCl was added. The animals used were from Woodruff's stock pedigree culture of *P. calkinsi*.

It was found, however, that when distilled water was used in making the hay infusion, the cultures of *B. pyocyaneus* made from it were not toxic to *P. calkinsi*. During the summer of 1926, repeated tests for toxic effects were made by introducing lines of *P. calkinsi* into such cultures of *B. pyocyaneus*, and in no case was there an evidence of toxic action. Care was taken to prepare these cultures in the same manner as all toxic cultures had previously been made, except that distilled water was used instead of tap-water. When, finally, it was evident that the distilled-water cultures were non-toxic to the lines of *P. calkinsi*, the same lines of animals were introduced into cultures of *B. pyocyaneus* made from St. Louis tap-water, and the usual toxic effects were obtained. In the cultures of *B. pyocyaneus* made from the St. Louis tap-water all of the lines died out within a week, in spite of the fact that they had previously grown for a long period of time in the non-toxic cultures of *B. pyocyaneus* made with distilled water. The hydrogen-ion concentration of these cultures as determined by means of the hydrogen-electrode potentiometer were 8.2 for the toxic and 7.67 at one time and 8.2 at another for the non-toxic. Since, by this method of determining hydrogen-ion concentration, all CO_2 is driven out of solution, it is probable that these cultures were less alkaline than the above readings indicate. The same lines of animals and the same strain of *B. pyocyaneus* were used in these toxic and non-toxic cultures, so that it is improbable that the difference was due to variations in these organisms.

Later, results similar to those described for *P. calkinsi* were obtained for *P. caudatum* and *P. aurelia*. Two experiments were carried out with each of these species where, in

each case, four lines were grown in cultures of *B. pyocyaneus* made from distilled water, four in cultures of *B. pyocyaneus* made from tap-water, and four in chance-mixed bacterial cultures. In each experiment the four lines in the cultures of *B. pyocyaneus* made from tap-water died within from one to seven days, while all other lines continued to live (tables 10 and 11). No reason for the non-toxic action of the cultures in distilled-water media is apparent unless it is due to the absence of certain buffer substances in the distilled water that were present in tap-water.

DURATION OF THIS ACQUIRED TOLERANCE

The tolerance acquired by a race of paramecia to the toxic action of a culture of *B. pyocyaneus* is not permanent. On March 22, 1926, four tolerant lines of *P. aurelia*, which had previously grown for fifteen days in toxic cultures of *B. pyocyaneus*, were introduced into chance-mixed bacterial cultures and grown there for twelve days or for what amounted to an average of fourteen generations in the four lines. The four lines were then returned to the toxic cultures of *B. pyocyaneus* in which they died within three days. Evidently, the tolerance which the lines had previously acquired was lost during the period of growth in the mixed bacterial cultures. During all of this time four sister tolerant lines continued to grow in the toxic infusions at a high division rate with no deaths. These results are shown in figure 6. The four tolerant lines referred to here are the same as have been described in connection with figure 4 of this paper.

A repetition of this experiment is also shown in figure 6. On May 2nd, from the same four tolerant lines of *P. aurelia*, four lines were introduced into chance-mixed bacterial cul-

Fig. 6 Results of an experiment to determine the duration in races of *P. aurelia* of the acquired tolerance to the toxic action of cultures of *B. pyocyaneus*. The heavy marking represents the average division rate of four tolerant lines of *P. aurelia* growing in two-day cultures of *B. pyocyaneus* from February 26 to June 5, 1926. The division rates have been averaged for five-day periods. Each of the dotted markings represents the fate of four of the tolerant lines which were grown in chance-mixed bacterial cultures for a period of time and then returned to the toxic cultures of *B. pyocyaneus*.

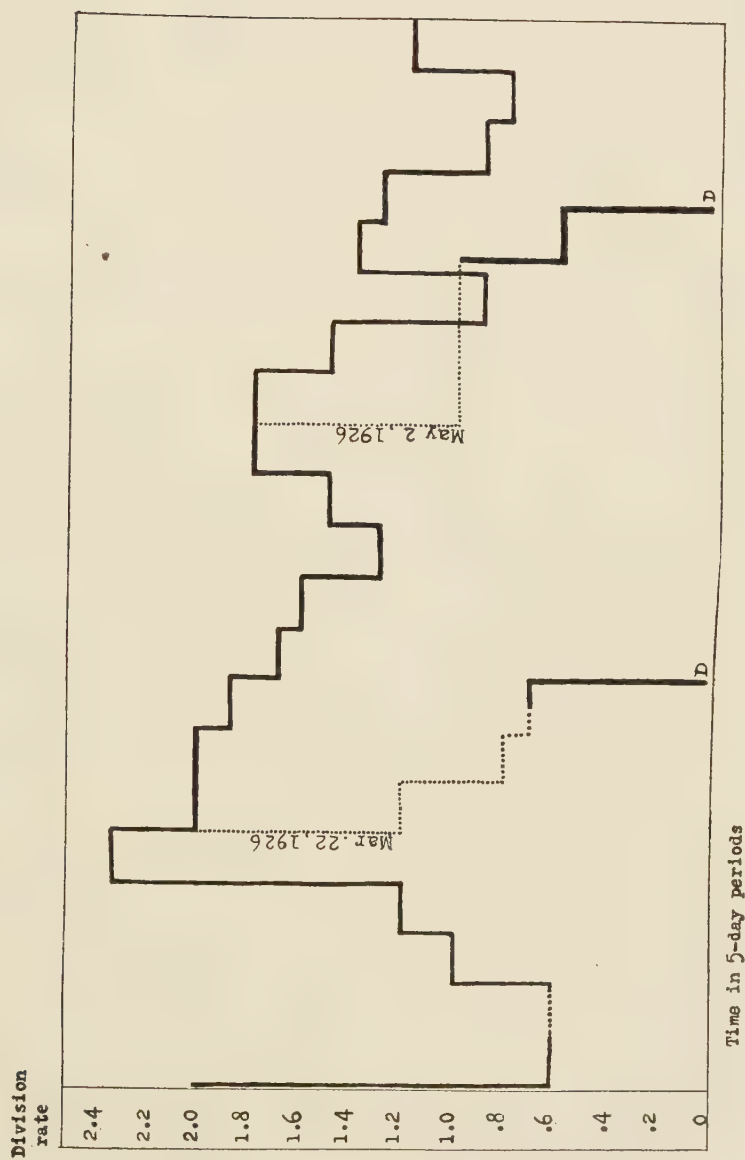


Figure 6

tures and grown there for seventeen days. During this period an average of ten generations occurred in the four lines. These four lines were then returned to cultures of *B. pyocyaneus*, in which they died within six days. During the entire time of the experiment four sister tolerant lines continued in the toxic cultures with no fatalities. It is here evident again that the acquired tolerance was lost, this time during a period of seventeen days' growth, or ten generations, in mixed bacterial cultures.

NATURE OF THE TOXIC AGENT IN CULTURES OF *BACILLUS PYOCYANEUS*

There are several substances known to be present in cultures of *Bacillus pyocyaneus*, any one of which might produce a toxic effect upon paramecium. Wasserman ('96) concluded from the result of his investigations that this organism produces both a soluble exotoxin and an endotoxin. Although the strength of the exotoxin never approaches that of diphtheria and tetanus, the blood of the animals immunized against it contains antitoxic substances. Acklin ('25) has shown that various strains of *B. pyocyaneus* growing in relatively simple, albuminoid-free media retain their virulence and ability to produce toxin. Wasserman ('96) reported that animals actively immunized with living culture masses of *B. pyocyaneus*, that is with the endotoxin, develop only bacteriolytic antibodies in their blood serum.

Emmerich and Low ('99) report that filtrates of old broth cultures of *B. pyocyaneus* contain a ferment-like substance which they call 'pyocyanase.' This is able to destroy other bacteria, apparently by lysis. They describe pyocyanase as being exceedingly thermostabile, since it resists boiling for several hours.

Bulloch and Hunter ('00) report the presence in cultures of *B. pyocyaneus* of another thermostabile substance which they called 'pyocyanolysin.' This agent is capable of producing a hemolyzing effect. Jordan ('03) thought, however, that this hemolyzing effect was due to the alkalinity of the filtrates.

Petty ('21) has shown that *B. pyocyaneus* growing in egg-broth gelatin and in a synthetic broth produces measurable quantities of hydrocyanic acid. He found that the amount varied with different strains of *B. pyocyaneus* and with the media and the pH.

The attempt has been made in the present study to use the method of elimination as the means of determining which, if any, of the above agents may be responsible for the toxic action of cultures of *B. pyocyaneus* upon paramecia. It was recognized at the outset that the reliability of this method depends upon the extent to which all possible causative agents are taken into consideration. There may, of course, be other toxic agents in cultures of *pyocyaneus* in addition to those above cited; but, in view of the many studies which have been made relative to the toxic action of this organism, the existence of such agents seems improbable.

The results obtained indicate that it is something dissolved in the cultures of *B. pyocyaneus*, and not the endotoxin which serves as the toxic agent. Attention has already been called to the fact that cultures of this organism in hay infusion made from distilled water were not toxic to any of the three species of paramecia used in this study. Neither did races of paramecia grown in non-toxic cultures of *B. pyocyaneus* develop any resistance that enabled them to grow in toxic cultures of these bacilli. Since paramecia growing in these non-toxic cultures are feeding upon the living bacilli, it would seem that the endotoxin is not the toxic agent. Otherwise one must suppose that the endotoxin is non-toxic under one set of conditions and toxic under the other. The conclusion that the endotoxin is not responsible for the toxic action is further strengthened by the fact that filtrates of cultures of *B. pyocyaneus* have been found to contain a soluble toxic agent.

That an agent toxic to paramecium is produced in the medium in which *pyocyaneus* bacilli grow is indicated by the results of experiments which show that tolerance in paramecia may be developed by subjecting the animals to the

effects of filtrates of cultures of *B. pyocyaneus*. Table 6 shows the results of one of these experiments. For use in the experiments filtrates of cultures of *B. pyocyaneus* were made by passing two-day cultures of this organism through Berkefeld filters. Four lines of *P. aurelia* were grown in these filtrates as shown in table 6. On August 14th, the animals were grown in the filtrate for a twenty-four-hour

TABLE 6

Development of tolerance by P. aurelia to growth in two-day cultures of B. pyocyaneus. Numbers in parentheses are the divisions for the day in filtrates of cultures of B. pyocyaneus. The italic numbers represent divisions per day in two-day cultures of B. pyocyaneus. Numbers without parentheses or italics represent divisions in chance-mixed bacterial cultures. The four lines (A) were rendered tolerant by growing during four different days in filtrates of B. pyocyaneus. Four sister control lines (B) were grown during the time in chance-mixed bacterial cultures and were not subjected to the filtrate. These four lines, when introduced into cultures of B. pyocyaneus, could not resist its toxic action and died as shown on the days marked 'D' in the table

AUGUST

	14	15	16	17	18	19		20	21	22	23	24	25	26	27	28	29	30		
(A)	{	(0)	1	1	(0)	(2)	(1)	Transferred to two-day cultures of <i>B.</i> <i>pyocyaneus</i>	1	2	2	2	2	2	3	1	2	2	3	Continued in mass cultures
		(0)	1	1	(0)	(D)	(1)		1	2	2	2	3	2	3	0	2	3	3	
		(0)	1	2	(0)	(0)	(1)		1	1	2	2	0	2	3	2	2	2	2	
		(0)	1	1	(1)	(1)	(1)		2	2	3	3	3	2	3	2	2	2	3	
(B)	{	1	0	2	1	0	1	Transferred to two-day cultures of <i>B.</i> <i>pyocyaneus</i>	1	1	2	0	0	1	0	0	0	D		
		1	0	D	2	0	1		2	0	0	0	1	0	0	0	D			
		1	0	1	1	0	2		2	2	1	1	1	1	0	1	1	0	D	
		1	0	2	1	0	1		2	0	1	0	1	1	0	0	0	0	D	

period. This was accomplished by placing the sterile filtrate in the cavities of depression slides contained in Petri dishes and introducing with each animal barely enough miscellaneous bacteria to supply food. The four lines were then allowed to recover for a two-day period in chance-mixed bacterial cultures, after which they were again grown in filtrates of *B. pyocyaneus* for three days. At the end of this period, the four lines were introduced into two-day cultures of *B. pyocyaneus* (table 6), where they were able to maintain a high division rate. The four control sister lines, which were not

grown during the first six days in the toxic filtrates, but in non-toxic mixed bacterial cultures, were unable, when introduced into cultures of *B. pyocyaneus*, to resist the toxic effect. It thus appears that the four lines subjected at intervals during a six-day period to filtrates of *B. pyocyaneus* acquired a tolerance to the toxic agent. Unfortunately, it was impossible to continue the four tolerant lines by the daily isolation method after August 30th. Mass cultures were made, however, and these continued for three weeks. All the lines of paramecia used in the experiment, including the controls, descended from one animal isolated at the beginning. After the sixth day, all tolerant and control lines were grown each day in portions of a culture of *B. pyocyaneus* removed from one flask. This served to keep the environment the same for all lines. The temperature was controlled to between 24° and 28°C.

A similar experiment was performed with *P. caudatum*, the results of which are shown in table 7. Here the results are not so satisfactory. The four lines that were grown at intervals in filtrates of *B. pyocyaneus* and then introduced into cultures of this organism were, as expected, tolerant to the toxic action. Two of the control lines which had been subjected to the filtrates died in accordance with expectations shortly after being introduced into the toxic cultures. The other two control lines survived. The fact that these two surviving control lines went through a critical period during the first six days of their growth in the toxic cultures may be significant. During the third, fourth, and fifth days there were no divisions in either of these lines. During the sixth day in the cultures of *B. pyocyaneus* there was only one division in each of these lines. During the same period of time the four sister tolerant lines, as shown in table 7 (A), were growing in the same cultures of *B. pyocyaneus* and at a high division rate. A depression period seems, therefore, to have occurred in these control lines when they were introduced into the toxic cultures. After the first six days in the toxic cultures, the two surviving control lines increased their division rates. My interpretation of these data is that the two control

lines of *P. caudatum* were able to survive and recover from the toxic effects of the cultures of *B. pyocyaneus* and that they acquired a tolerance for the toxic substance in the culture as a result of their experience. The method used in rendering lines of *P. caudatum* tolerant by means of toxic filtrates were the same in every way as those used with *P. aurelia* and described above.

TABLE 7

Development of tolerance by P. caudatum to growth in two-day cultures of B. pyocyaneus. Numbers in parentheses are the divisions for the day in filtrates of cultures of B. pyocyaneus. The italic numbers represent divisions per day in two-day cultures of B. pyocyaneus. Numbers without parentheses or italics represent divisions in chance-mixed bacterial cultures. The four lines (A) were rendered tolerant by growing during three different days in filtrates of B. pyocyaneus. Four sister control lines (B) were grown during the time in chance-mixed bacterial cultures and were not subjected to the toxic filtrates. Two of these lines when introduced into the cultures of B. pyocyaneus could not resist its toxic action and died as shown on the days marked 'D' in the table. The other two lines continued to grow after a period of depression

AUGUST																																		
		14	15	16	17	18	19	20	21			22	23	24	25	26	27	28	29	30														
(A)	{	2	0	2	(0)	1	(1)	(0)	0	Transferred to two-day cultures of <i>B. pyocyaneus</i>		3	3	3	3	4	4	3	4	3	Continued in mass cultures													
		0	D	2	(1)	0	(1)	(1)	0			3	3	2	3	4	3	4	4	3														
		0	1	1	(0)	0	(0)	(0)	1			2	3	3	3	3	3	4	4															
		3	0	2	(0)	0	(1)	(0)	1			3	3	4	3	4	4	4	4															
(B)	{	2	0	2	1	1	3	4	0	Transferred to two-day cultures of <i>B. pyocyaneus</i>		3	0	1	D						Continued in mass cultures													
		0	D	2	1	1	2	4	0			2	0	D																				
		0	1	1	2	1	2	2	0			1	2	0	0	0	1	3	4	3														
		3	0	2	1	1	3	3	0			2	2	0	0	0	1	3	4	3														

It has been impossible to demonstrate directly that the filtrates of *B. pyocyaneus* are toxic for paramecia. The filtrate when first obtained is, of necessity, sterile. When paramecia are introduced into this sterile filtrate, they die of starvation, unless miscellaneous bacteria are added as food. Thus it is impossible to determine directly whether the death of an animal that has been introduced into the supposedly toxic filtrate is a result of toxic action or of starvation.

It appears from another experiment that the toxic agent in cultures of *B. pyocyaneus* is destroyed by heat (table 8). In this experiment eight lines of *P. aurelia* were grown from

TABLE 8

Results of an experiment to determine whether cultures of B. pyocyaneus that had been heated had lost their ability to induce the development of tolerance in lines of P. aurelia. May 2 to May 19, 1926. Each figure represents the number of divisions for the day

Growth for fourteen days in cultures of *Bacillus pyocyaneus* which had been heated to 80°C. for fifteen minutes

(A)	{	2	1	1	2	2	1	1	2	2	1	1	1	2	1	Transferred to two-day cultures of <i>B.</i> <i>pyocyaneus</i>	2	0	D
		2	1	2	1	3	2	2	2	0	1	1	1	1	1		3	D	
		2	1	2	1	3	2	2	0	2	2	1	1	1	1		2	0	D
		0	1	2	1	2	1	1	1	1	1	2	2	1	1		2	D	

Growth for fourteen days in cultures of *B. pyocyaneus* which had been heated to 100°C. for fifteen minutes

(B)	{	2	1	1	2	1	1	2	2	2	1	2	1	1	1	Transferred to two-day cultures of <i>B.</i> <i>pyocyaneus</i>	3	0	0	D	
		2	1	2	1	2	1	2	2	2	1	1	1	2	1		0	0	D		
		2	1	2	1	1	1	1	2	2	2	1	1	1	1		0	0	1	0	D
		0	1	1	1	1	1	2	2	2	2	0	2	2	1		2	0	0	0	D

Growth for fourteen days in chance-mixed bacterial cultures

(C)	{	2	2	1	1	1	2	0	0	D	2	1	0	0	2	Transferred to two-day cultures of <i>B.</i> <i>pyocyaneus</i>	2	D		
		2	3	2	1	1	2	0	0	0	1	2	0	1	1		2	D		
		2	2	2	1	2	0	1	0	2	0	0	0	1	1		2	D		
		2	2	2	1	1	2	1	D	D	2	2	1	1	1		2	0	0	D

Growth of control tolerant sister lines in two-day cultures of *B. pyocyaneus*

(D)	{	2	1	2	2	2	2	2	2	0	2	1	1	1	Continued in two-day cultures of <i>B.</i> <i>pyocyaneus</i>	3	2	1	2	2	1	1	
		2	1	2	2	2	1	2	2	1	2	1	1	1		1	2	2	1	2	2	1	2
		2	2	1	2	1	0	2	2	2	0	1	1	0		1	0	1	0	1	1	0	1
		3	2	2	1	1	1	1	2	0	1	1	1	1		1	1	2	2	1	1	1	2

day to day in cultures of *B. pyocyaneus* that had been heated to a temperature high enough to kill all bacilli and to destroy anything in the nature of a toxin. The temperatures used were 80° and 100°C. for fifteen minutes. These lines of paramecia were carried by means of the daily isolation

method for a period of fourteen days in the boiled cultures of *B. pyocyaneus*. In transferring each animal into the culture that had been heated, enough miscellaneous bacteria were carried along with the paramecium to provide food for the following day, but care was taken to keep this bacterial contamination at a minimum. The results given in table 8 show no evidence that the heated cultures were toxic to paramecia. The lines growing in these heated cultures (A and B) displayed division rates as high as those for the control lines (C) in the chance-mixed bacterial cultures. At the end of the fourteen-day period the eight lines of paramecia were introduced into two-day cultures of *B. pyocyaneus*. Each of these lines died in a few days after introduction into the toxic cultures, which shows that there was nothing in the heated culture that had the effect of stimulating the development of tolerance in the race of paramecia. During the time that these lines were dying in the toxic cultures of *B. pyocyaneus*, four control tolerant sister lines (D) grew day by day in the same toxic cultures used for the dying lines and with no depression in division rate. This indicates that a toxic agent was responsible for the death in all lines indicated in A, B, and C. The control tolerant lines shown in D of this figure are the same as those described in figure 4 of this paper.

The fact that a boiling temperature for fifteen minutes destroys the effectiveness of the toxic action in a culture of *B. pyocyaneus* eliminates the possibility of this action being due to the presence of either pyocyyanase or pyocycyanolysin, because these substances are able to resist boiling for several hours (compare p. 108).

The experiment showing the effect of heat upon the toxic agent does not, however, eliminate hydrocyanic acid as the responsible factor, since boiling for fifteen minutes might drive off this volatile substance. There are, however, indications that HCN is not responsible for the toxic action of *B. pyocyaneus* upon paramecia. In the first place, it is probable that very little of the HCN, known to be produced in the cultures of *B. pyocyaneus* used in these experiments, could have

existed in the free state, since these cultures had a reaction on the alkaline side of neutrality. In such a medium as alkaline hay infusion it is probable that much of the free HCN was converted into salts which are not so toxic as free HCN.³ In the second place, certain facts indicate the absence of any correlation between the degree of toxic action of certain cultures and the amount of HCN they were found to produce in a given time. Indeed, certain cultures of *B. pyocyaneus* found to be non-toxic to paramecia produced in a given time as much HCN as was produced in toxic cultures (table 9).

The method of estimating the amount of HCN was that used by Petty ('21). In each determination 200 cc. of hay

TABLE 9

Estimated amount per cubic centimeter of HCN liberated in seven days in cultures of B. pyocyaneus made from distilled-water hay infusions and from tap-water hay infusions. Each culture was incubated for seven days at 35°C. The pH determinations were made by colorimetric methods

	DISTILLED-WATER CULTURES	pH	TAP-WATER CULTURES	pH
First determination	.00033 mg.	7.0	.00018 mg.	8.0
Second determination	.00044 mg.	7.0	.00041 mg.	8.5

water in a 250-cc. flask was inoculated with *Bacillus pyocyaneus* and incubated for seven days at 35°C. During this period air, filtered through soda lime, was passed through the culture and into 4 per cent KOH. At the end of this seven-day period the KOH solution was added to the seven-day culture of *B. pyocyaneus*, the whole was acidified with sulphuric acid and distilled into 4 per cent KOH. To the distillate 1 cc. of yellow ammonium sulphide was added, the whole was evaporated to dryness on a water-bath and the residue was extracted with three 10-cc. portions of acetone. The acetone solution was then evaporated to dryness and water added. To this solution of potassium sulphocyanide fifteen drops of 5 per cent ferric chloride solution was added,

³ Sollman, Torold. *A Manual of Pharmacology*, third edition, 1926, p. 774.

which converted the KCNS into blood-red ferric thiocyanate. The amount of the latter was determined colorometrically by comparing with freshly made KCNS solutions of known strength to which ferric chloride solution had been added.

The results shown in table 9 indicate that the amount of HCN present in the toxic two-day cultures of *B. pyocyaneus* used in this study was very small. Especially is this evident when one considers the fact that the amount shown in the table represents the total amount produced in a culture of *B. pyocyaneus* in seven days, and not the amount present at any one time; and that the estimated amount includes not only the free HCN present in the culture during the period, but also the HCN derived from the salts of cyanide that were formed in the culture. As has been pointed out, all of the hay-infusion cultures of *B. pyocyaneus* used in this study had a pH on the alkaline side of neutrality. It is probable that much of the HCN in each culture was converted into salts, as fast as it was formed, with the result that little of it existed at any one time in its free state.

Table 9 also indicates that a greater amount of HCN was present in the hay-infusion cultures of *B. pyocyaneus* that were made with distilled water than in those made with tap-water. Since the distilled-water cultures of *B. pyocyaneus*, in which the greater amounts of HCN existed, were non-toxic to paramecia, while similar cultures made from tap-water were toxic, it would seem that HCN is not the toxic agent. One may conclude that there is no correlation between the toxicity and the amount of HCN present under the two conditions, because the amount of HCN present in the non-toxic cultures is, if anything, slightly more than in the toxic cultures.

This non-toxic action of cultures of *B. pyocyaneus* made from distilled water was first observed in connection with the growth of *P. caulkinsi* in cultures of *B. pyocyaneus* as has been previously explained (p. 105). To make sure of this difference in toxicity in the two types of cultures further experiments were undertaken with *P. caudatum* and *P. aurelia*. The

determinations were being made. The distilled-water hay infusions used in making the cultures of *B. pyocyaneus* for the HCN determinations and the cultures of *B. pyocyaneus* in which the races of paramecia were grown were made up at one time in one large container. Likewise, the tap-water infusions used for both purposes were made in one container. Thus there could have been little variation of media in the similar cultures used for the different purposes.

It is evident from the results presented in tables 10 and 11 that the cultures of *B. pyocyaneus* which were made from tap-water hay infusions and which, as shown in table 9, contained the smaller amount of HCN, were toxic to *P. aurelia* and *P. caudatum*, while similar cultures which were made from distilled-water hay infusions and which contained a greater quantity of HCN were non-toxic.

The alkalinity or acidity of the hay-infusion cultures is not responsible for the toxic action upon paramecia. The hay-infusion cultures of *B. pyocyaneus* which were toxic to *P. aurelia* had a pH of from 8.2 to 8.5. The cultures that were toxic to *P. caudatum* had a pH of 7.0, while the non-toxic, mixed bacterial cultures, used as controls, had a pH ranging from 7.1 to 8.0. The cultures of *B. pyocyaneus* that were toxic to *P. caudatum* had a pH of 7.0, while the non-toxic, mixed bacterial cultures, used as controls, had a pH of 6.8. These determinations were made by colorimetric methods.

It may also be mentioned that the agency in cultures of *B. pyocyaneus* that is toxic to paramecia does not cause immediate death of these animals. Death in any line usually results after a period of from five to ten days of growth in the toxic cultures, during which a high division rate of the paramecia is not uncommon. This may be compared with the 'period of incubation' of a toxin or time elapsing between inoculation and death as seen in higher animals. Thus a guinea-pig usually dies in or after the fourth day following inoculation with a lethal dose of diphtheria toxin, and such incubation periods are a distinguishing feature of toxins.

The facts above presented seem to eliminate the possibility that the toxic action of the cultures of *B. pyocyaneus* is due to endotoxin, to pyocyanase, to pyocyanolysin, to hydrocyanic acid, or to hydrogen-ion concentration. They point to the soluble toxin of *B. pyocyaneus* as being the toxic agent unless there is some other poisonous substance produced in the culture by the bacillus that is either thermolabile or capable of being volatilized and driven out by boiling temperatures.

In the absence of exact knowledge regarding the chemical nature of what are called 'toxins,' it is perhaps as justifiable to call the lethal agent a 'toxin' as a 'deleterious substance,' and to conclude that an 'immunity' to *B. pyocyaneus* can be developed in paramecium comparable with that produced in higher animals. Such was the terminology used in the preliminary reports of the present work (Philpott, '25, '26). The subsequent discovery of Petty's account ('21) of the production of hydrocyanic acid by *Bacillus pyocyaneus* has led to a more cautious statement, although it does not seem that hydrocyanic acid is the effective agent. It has seemed advisable, therefore, to use the term 'toxic substance' and 'tolerance' throughout, although it is probable that we are dealing with a true case of immunization against a toxin. Similar results with other toxins and with other protozoa are needed to establish the conclusion that races of protozoa may be immunized to toxins.

GROWTH OF PARAMECIA IN PURE CULTURES OF BACILLUS ENTERITIDIS

In contrast with the foregoing relationships in cultures of *Bacillus pyocyaneus*, the evidence thus far obtained indicates that paramecia will not grow in pure cultures of *Bacillus enteritidis*. All attempts that have been made to render pure lines of paramecia capable of growth in pure cultures of *B. enteritidis* have failed.

Bacillus enteritidis Gaertner was selected as an organism to be used in this study, because of its pathogenic effects. It is perhaps the commonest cause of food poisoning and affects

a wide variety of vertebrate animals. The poisonous product of the growth of this organism is known to be an endotoxin, and not a true toxin. No antitoxic substances are known to be developed. The strain of the organism used was obtained from the American Type Culture Collection of 637 S. Wood Street, Chicago, Illinois.

TABLE 12

Growth of twelve lines of Paramecium caudatum in pure cultures of Bacillus enteritidis. The number of days indicated is the entire time the line grew in cultures of B. enteritidis before dying out. The division rate shown for the control lines in the mixed bacterial cultures is the average number of divisions per day for the number of days during which the line in the cultures of B. enteritidis continued to grow. In each case the animals in the control belonged to the same pure line as those in the cultures of B. enteritidis

NO.	DATE	NUMBER OF DAYS	AVERAGE DIVISION RATE PER DAY	AVERAGE DIVISION RATE IN CONTROL
1925				
1	February 24	5	.4	.6
2	February 24	5	.4	.6
3	March 5	2	.6	1.5
4	March 5	3	1.0	1.0
5	March 4	3	1.0	1.5
6	March 4	4	1.0	1.5
7	March 5	2	.5	1.5
8	March 5	3	.3	1.0
9	March 12	2	.5	1.0
10	March 12	2	.5	1.0
11	March 13	4	.5	2.0
12	March 13	4	.5	2.0
Mean		3.25	.6	1.26

The cultures of *B. enteritidis* were made by inoculating 0.2 per cent sterile hay water contained in cotton-plugged flasks and incubating for two days. Quantitative bacteriological analyses indicated that the bacilli grew profusely in the medium.

The animals used belonged to a pure line of *P. caudatum* descended from one individual isolated from Saint Louis pond water on February 23, 1925.

The results of the growth of twelve typical cases of *P. caudatum* in enteritidis and in mixed cultures are shown in table 12. It appears that the cultures of *B. enteritidis* are toxic to *P. caudatum*. The lines of paramecia were not killed immediately when introduced into the cultures, but continued to live for an average of 3.25 days. During the period when the lines in the cultures of *B. enteritidis* were dying the sister control lines in the mixed cultures were growing normally, showing that death in the toxic cultures was due either to the condition of the medium or to the nature of the food. The average division rate for the twelve lines in the cultures of *B. enteritidis* was 0.6, as compared with an average of 1.26 for the control lines in the chance-mixed bacterial cultures.

Attempts were made without success to habituate lines of *P. caudatum* to growth in cultures of *B. enteritidis*. No tolerance was developed in the lines even when three recovery periods were provided. Neither was it evident that the lines developed any greater degree of sensitiveness to enteritidis bacilli as a result of the periods of growth and recovery.

GROWTH OF PARAMECIA IN MEDIA CONTAINING DIPHTHERIA TOXIN

In the preceding section attention has been called to the difficulty of determining whether the resistance acquired by races of paramecia to the toxic action of cultures of *B. pyocyaneus* represents an acquired tolerance to a poison or a true case of immunization to a toxin. It would be easier to study the phenomenon of immunization in races of paramecia, if such exists, by working with standard toxins. Minute and measured amounts of such material could be added to culture media of known composition, and if toxic action resulted there could be no question about the causative agent. At the present stage of this investigation, diphtheria toxin is the only one of this type that has been employed.

The diphtheria toxin used was supplied through the kindness of Dr. Mazyck P. Ravenel, of the Department of Medical Bacteriology and Preventive Medicine, and director of the

Public Health Laboratory of the University of Missouri, for whom it was prepared and tested by the Gilliland Laboratories, Marietta, Pennsylvania. The toxin, of necessity, differed from the usual preparations, since it was devoid of preservatives added to prevent bacterial growth. The presence of the preservative would doubtless have had an effect upon the growth of paramecia, and consequently would have complicated the study. The toxin used throughout the study had an M. L. D. of 300, i.e., $\frac{1}{300}$ cc. was the minimum amount required to kill a guinea-pig weighing 250 grams in four days.

The experimental animals were in each case grown from day to day in 0.2 per cent timothy-hay infusion to which measured quantities of toxin had been added. The timothy-hay infusion was prepared as has been previously described and enough was made at one time to last throughout the experiment. This infusion after sterilization had a pH of 7.32. The medium for each day was prepared by adding 0.12 cc. of the diphtheria toxin to 20 cc. of the sterile hay infusion. For the growth of each animal 0.5 cc. of this mixture was added to the cavity of a culture slide in a Petri dish and 0.2 cc. of a twenty-four-hour mixed-bacterial culture in hay infusion was added for food. Thus there was provided in each cavity of a culture slide for each paramecium a total of $\frac{1}{333}$ cc. of diphtheria toxin—almost a minimal lethal dose for a guinea-pig weighing 250 grams. It was found that when a greater amount of toxin was added the presence of the nutritive materials in the added broth containing the toxin brought about the development in the hay infusion of bacterial scums and precipitates that hindered the normal growth of the paramecia and complicated the experiment by increasing the possibility of the active toxin being modified or destroyed by the numerous bacteria and their products. It was intended to provide in the medium the maximum amount of diphtheria toxin that could be used without causing the formation of such bacteriological scums and precipitates. After many trials it was found that the mixture of 0.12 cc. of toxin to 20 cc. of the hay water was the most satisfactory.

That detoxification did not occur, when diphtheria toxin was added to the hay water, was determined by injecting guinea-pigs with fatal doses of the toxin diluted in 0.2 per cent hay infusion. That the presence of bacteria added to the hay infusion-toxin mixtures did not modify or destroy the toxin is indicated by the following experiment. On August 30th, a guinea-pig weighing 380 grams was injected with 1.5 cc. of a hay infusion-toxin mixture. This time 0.12 cc. of the toxin was added to 10 cc. of sterile 0.2 per cent hay infusion, and the mixture was then heavily inoculated with air bacteria and inoculated at 25°C. for six hours. At the end of this time, the contaminated hay infusion-toxin mixture was passed through a Berkefeld filter and 1.5 cc. of the sterile filtrate injected intraperitoneally into the guinea-pig. The animal died in forty-eight hours. Two control animals each injected with filtrate similar in every way to that described above, except that the toxin had been omitted, remained alive.

Pure lines of three species of paramecia were used in this study. The pure line of *P. caudatum* came from an individual isolated from an aquarium in Columbia, Missouri, on August 7, 1926, and the lines of *P. aurelia* and *P. calkinsi* from individuals isolated from Woodruff's pedigreed stocks.

In each experiment undertaken one animal was isolated and its immediate descendants were used as the beginning of, *a*) four lines to be grown from day to day in cultures containing diphtheria toxin; *b*) four lines in cultures containing boiled toxin, and, *c*) four lines in cultures containing no toxin. As usual, the daily isolation method was used and records were kept of the number of divisions per day. Hay infusion-toxin mixtures were made each day just before the transfer of animals to fresh media. The temperature of all infusions containing paramecia was controlled to between 23° and 25°C., since the infusion-toxin mixtures at higher temperatures often developed troublesome scums and precipitates.

It was found that lines introduced into cultures containing toxin as well as into cultures containing boiled toxin did not

survive many days. Wherever a line died out in such a culture, a new line was started to take its place. Such new lines were in every case started from individuals discarded from the control lines growing in the culture containing no toxin. Thus, while there were only four lines under observation for each kind of media at any one time, the total number of lines used during the entire time of the experiment in the culture that contained toxin and in the cultures that contained boiled toxin was greater than four. At the end of each experiment it was possible to determine for each type of medium used the total number of lines introduced and the average duration of life in each line.

TABLE 13

Effect of diphtheria toxin on lines of P. caudatum. Duration of experiment, sixteen days

	TOTAL NUMBER OF LINES INTRODUCED INTO THE MEDIUM	AVERAGE NUMBER OF DAYS LINES SURVIVED	PERCENTAGE OF DEATHS	MEAN DIVISION RATE
A. Medium containing toxin	9	8.3	10	1.08
B. Medium containing boiled toxin	7	8.0	8	1.17
C. Medium containing no toxin	4	16.0	1	.73

The data obtained with respect to *P. caudatum* are shown in table 13. It is evident from a consideration of the items in the table that, under the conditions of the experiment, diphtheria toxin had no appreciable effect upon this species of paramecium. The average number of days the lines (A) survived in the presence of the toxin was 8.3, as compared with 8.0 for the lines (B) growing in the presence of boiled toxin. The mean division rate for the lines (A) growing in the presence of toxin was 1.08, as compared with 1.17 for lines (B) growing in the presence of boiled toxin. There was, similarly, no difference of any significance in the percentage of deaths in the lines under the two experimental conditions, since it was 10 per cent for the former and 8 per cent for the latter.

The death rate and division rate in the four lines in (C) that were grown in the media containing no toxin is quite different from that for the lines in (A) and (B). This difference is due to the absence in (C) of certain nutritive substances which were present in the toxin added to all media used for lines in (A) and (B). These nutritive substances include peptone, beef extract, and dextrose in small quantities.

TABLE 14

Effect of diphtheria toxin on lines of P. aurelia. The experiment covered a period of nineteen days, from August 11 to August 30, 1926. Twelve sister lines of paramecia were started in the infusions, four in A, four in B, and four in C. In each case where a line died out another was started to take its place. Such replacements in every case were made from animals discarded from line C. Each replacement thus made constituted the beginning of a new line and the total number of lines introduced into each medium throughout the time of the experiment is shown in the first column

	TOTAL NUMBER OF LINES INTRODUCED INTO MEDIUM	AVERAGE NUMBER OF DAYS LINES SURVIVED	PROBABLE ERROR	PERCENTAGE OF DEATHS	MEAN DIVISION RATE	PROBABLE ERROR
A. Medium containing toxin	13	5.8	3.5	13	1.0	.2
B. Medium containing boiled toxin	7	10.0	3.6	6	1.0	.2
C. Medium containing no toxin	5	16.5	1.6	4	.9	.5

The results for *P. aurelia*, as shown in table 14, might seem to indicate that the diphtheria toxin had some effect upon metabolism in this species. However, when one considers the large probable error for some of the figures in the table it becomes evident that the conclusion may also be made here that the presence of the toxin had no appreciable effect upon the division rate or upon the time that a race of these animals is able to survive in the infusions. It is particularly noticeable that the percentage of deaths in the lines of *P. aurelia* growing in the presence of a toxin was 13 as compared with a percentage of 6 for the lines in the presence

of the boiled toxin. Here it might seem that the presence of the toxin had some influence upon the death rate in the lines of *P. aurelia*. But the results obtained with *P. caudatum*, as shown in table 13, and with *P. calkinsi*, as shown in table 15, do not support this conclusion.

The results of a study of diphtheria toxin upon lines of *P. calkinsi*, as shown in table 15, justifies the same conclusion as with *P. caudatum* and *P. aurelia*, that the toxin had no appreciable effect upon the division or death rate of this species.

TABLE 15

Effect of diphtheria toxin on lines of Paramecium calkinsi. The experiment covered a period of twenty-four days, from August 6 to August 30, 1926. Twelve sister lines of paramecia were started in the infusions, four in A, four in B, and four in C. In each case where a line died out another line was started to take its place. Such replacements were, in every case, made from animals discarded from line C. Each replacement thus made constituted the beginning of a new line, and the total number of lines introduced into each medium is shown in the first column of the table

	TOTAL NUMBER OF LINES INTRODUCED INTO MEDIUM	AVERAGE NUMBER OF DAYS LINES SURVIVED	PROBABLE ERROR	PERCENTAGE OF DEATHS	MEAN DIVISION RATE
A. Medium containing toxin	7	13	6	4	.6
B. Medium containing boiled toxin	5	18	5	3	.6
C. Medium containing no toxin	4	23	.8	1	1.5

The results here obtained in the study of the relation of diphtheria toxin to growth in paramecia are similar to those obtained by Oehler ('21) on *Colpidium*. He grew three species of this organism in pure diluted bouillon cultures of *Bacillus diphtheria*. He worked with mass cultures of the protozoa and was not able to find any effects produced by the diphtheria cultures which were not produced in cultures of non-pathogenic bacteria. He was concerned, however, with cytological effects and not with effects upon the division rate.

DISCUSSION

It has been shown in the foregoing study that races of paramecia are able to adapt themselves to growth in toxic cultures of *Bacillus pyocyaneus*. The evidence indicates that this adaptation is an immunization to the soluble pyocyaneus toxin, although it is not established beyond question that it is not a case of acquired tolerance to some other deleterious substance. Whatever may be the fact as between the development of immunity or of tolerance, the adjustment of races of paramecia to growth in the presence of deleterious substances, as described in this investigation, differs from most studies that have been made of acclimatization in unicellular organisms, because the daily-isolation method has been used throughout. This eliminates the agency of selection. It is only by this method that one can hope to demonstrate the development in races of protozoa of either acquired tolerance or immunity as distinguished from the survival of tolerant strains that have originated in the mass independently of environmental influences.

In an attempt to demonstrate a true case of immunization in protozoa by growing the animals in cultures of toxin-producing bacteria, one is never sure that the deleterious effects of all toxic agents other than the 'toxin' have been eliminated. A better method of demonstrating a case of immunization in protozoa would be to find some standardized and purified toxin that affects paramecia and to add it in measured amounts to non-toxic hay infusions in which the protozoa grow. In the present investigation this was attempted only in the case of diphtheria toxin which was found not to have any appreciable effect upon paramecia.

The present study shows that paramecia may be grown in pure cultures of *B. pyocyaneus*. Even though it may be unwise to use any pathogenic bacillus extensively, the evidence here presented shows that paramecia may be grown in pure cultures of at least one species of bacteria. It is reasonable to hope that further investigation will reveal other species of bacteria, some of which may be non-pathogenic, that will be as serviceable in this respect as *B. pyocyaneus*.

SUMMARY

1. Cultures of *Bacillus pyocyaneus* are toxic under certain conditions to *Paramecium aurelia*, *P. calkinsi*, and *P. caudatum*, but pure-line cultures of these three species of paramecia may acquire a tolerance for this toxic agent.

2. Races of paramecia may be grown for long periods of time in pure cultures of *B. pyocyaneus*.

3. The data presented indicate that the toxic agent in cultures of *B. pyocyaneus* that is lethal to paramecia is probably the soluble toxin known to be produced by this bacillus.

4. Hydrocyanic acid was found in measurable quantities in hay-infusion cultures of *B. pyocyaneus*, but not in sufficient quantities to be injurious to paramecia.

5. Hay-infusion cultures of *Bacillus enteritidis* were lethal to paramecia. All attempts to develop tolerance in paramecia for the toxic agent in these cultures failed.

6. Under the experimental conditions that prevailed, diphtheria toxin had no appreciable effect upon the division rate or death rate of three species of paramecia.

7. The reliability of the method of washing paramecia until bacteria-free and of establishing and growing races of them in pure cultures of bacteria is discussed.

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VITA

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In addition to his Master's thesis, which was completed under the joint direction of the Departments of Zoölogy and Education of the University of Missouri and entitled "A course in elementary high school biology from within the students' environment," he has published the results of a study made at the Puget Sound Biological Station in 1922, entitled "Observations on the early development of *Argobuccinum oregonense*", in Publ. Puget Sound Biol. Sta., vol. 74 (1925), and has presented preliminary results of the present investigation before the American Society of Zoölogists, abstracts of which appear in The Anatomical Record, vol. 31, p. 314, and vol. 34, p. 156.

